(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 174 438 A1

(12) EUROPEAN PATENT APPLICATION
published in accordance with Art. 158(3) EPC

(43) Date of publication:
23.01.2002 Bulletin 2002/04

(21) Application number: 00905381.0

(22) Date of filing: 28.02.2000

(51) Int Cl.[7]: C07K 5/12, A61K 38/12

(86) International application number:
PCT/JP00/01141

(87) International publication number:
WO 00/52033 (08.09.2000 Gazette 2000/36)

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: 02.03.1999 JP 5385199

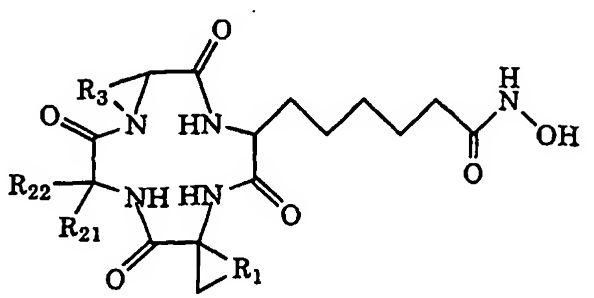
(71) Applicant: Japan Energy Corporation
Tokyo-to 105-8407 (JP)

(72) Inventors:
- Nishino, Norikazu
Kitakyushu-shi, Fukuoka 808-0104 (JP)
- Yoshida, Minoru
Kawaguchi-shi, Saitama 334-0059 (JP)
- Horinouchi, Sueharu
Koto-ku, Tokyo 135-0044 (JP)
- Komatsu, Yasuhiko, Japan Energy Corp.
Toda-shi, Saitama 335-8502 (JP)

(74) Representative:
Ziebig, Marlene, Dr. Dipl.-Chem. et al
Patentanwälte, Gulde Hengelhaupt Ziebig,
Schützenstrasse 15-17
10117 Berlin (DE)

(54) NOVEL CYCLIC TETRAPEPTIDE DERIVATIVES AND USE THEREOF AS DRUGS

(57) The present invention provides a cyclic tetrapeptide derivative represented by the following general formula (I) or a pharmaceutically acceptable salt thereof:



(I)

wherein

each of R_{21} and R_{22} independently denotes hydrogen, a linear C_1-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached, or a branched C_3-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached; and
each of R_1 and R_3 independently denotes a linear C_1-C_5-alkylene group which may have a C_1-C_6 side chain, in which the side chain may form a condensed ring structure on the alkylene chain.

The present invention also provides a histone deacetylase inhibitor, an MHC class-I molecule expression-promoting agent and a pharmaceutical composition, each of which comprises the above cyclic tetrapeptide derivative or pharmaceutically acceptable salt thereof as an active ingredient.

EP 1 174 438 A1

Printed by Jouve, 75001 PARIS (FR)

Description

TECHNICAL FIELD

[0001] The present invention relates to novel cyclic tetrapeptide derivatives or pharmaceutically acceptable salts thereof, application of said compounds as histone deacetylase inhibitors and MHC class-I molecule expression-promoting agents, as well as pharmaceutical compositions that comprise said cyclic tetrapeptide derivatives or pharmaceutically acceptable salts thereof as active ingredients and which have utility as pharmaceuticals such as anti-cancer agents by taking advantage of the above histone deacetylase-inhibiting or MHC class-I molecule expression-promoting action.

BACKGROUND ART

[0002] Individual's own tissue cells express on their cell surface an MHC class-I molecule as an antigen presenting molecule to discriminate externally invading foreign matters and pathogens from themselves, in order to prevent false damage by their immunocytes. The immune system recognizes the MHC class-I molecule to identify the self tissue cells and eliminate them from the target of its attack. On the other hand, cancerized cells or cells infected with cancer viruses, which are originally self cells, differ from normal self cells in that they produce proteins associated with canceration or proteins derived from the cancer viruses, and antigens derived from these non-self proteins are.presented by the MHC class-I molecule. The immunocytes, in particular cytotoxic T cells, can recognize the non-self protein-derived antigens, thereby eliminating the cancer cells or cancer virus-infected cells.

[0003] It has been reported, however, that in certain kinds of cancer cells or cancer virus-infected cells, the expression of the MHC class-I molecule is reduced, so that the above elimination mechanism by the immune system is circumvented, causing expansion and enlargement of cancerized tissues as well as prolonged sustention and enlargement of cancer virus infection. In the studies for the purpose of preventing tumorization of the cancerized cells or cancer virus-infected cells, some results have been reported suggesting that therapeutic effects may be attained by recovery of the reduced expression of the MHC class-I molecule. For example, Tanaka et al. reported that in cancer cells transformed with adenovirus 12 or spontaneous melanoma, tumorization of these cancer cells disappeared upon enhancing the reduced expression of the MHC class-I molecule through introduction of MHC class-I gene: see Tanaka, K., Isselbacher, K.J., Khoury, G. and Jay, G., Science, 228, 26-30, 1985; Tanaka, K., Gorelik, E., Watanabe, M., Hozumi, N. and Jay, G., Mol. Cell. Biol., 8, 1857-1861, 1988.

[0004] Meanwhile, the expression of MHC class-I molecule occurs during the differentiation process after the growth of the self tissue cells, and the expression of MHC class-I molecule is expected to be enhanced by promoting the translation of endogenous proteins in this process. While there are several mechanisms which control the translation of endogenous proteins, one of those which may be considered to play an important role in gene expression is acetylation of histone proteins contained in the nuclear gene chromatins as their structural proteins. Illustratively, chromatin is composed of the basic unit referred to as a nucleosome structure, in which a gene DNA is wound around four core histone octamers. Further, the basic units form higher-order structure. The neighborhood of the N-terminal of the core histone is in the form of a tail rich in basic amino acids and it further encloses the DNA on the above nucleosome. Lysine residues in the neighborhood of the tail region undergo reversible metabolic turnover of acetylation and are said to be closely involved in the structural control of nucleosome itself or in the transcriptional control through the control of binding with other proteins acting on gene DNA, such as transcriptional factors, silencer proteins and RNA polymerases.

[0005] As a demonstration of gene expression control depending on acetylation of histone, it has been reported that higher acetylation of histone promotes the induced expression from genes present in a region of interest while deacetylation forms a transcriptionally inactive region called heterochromatin. That is to say, histone which is a structural protein of chromatin and its acetylation are extended over the whole region of the chromosomal gene; nevertheless, it has been suggested that the function of histone greatly affects the expression of a specific gene and, in other words, is involved in the strict control of nuclear signal transmission. An enzyme for acetylating histone is histone acetyltransferase while an enzyme for deacetylating histone is histone deacetylase; these enzymes regulate the kinetic metabolic turnover relating to the level of histone acetylation.

[0006] If the action of histone deacetylase is enhanced, proper differentiation of cells or normalization of their morphology is inhibited; however, when the enzyme activity of the histone deacetylase is inhibited, the deacetylation from histone is inhibited and, as a result, high acetylation of histone is caused to induce the gene expression required for differentiation and normalization of cell morphology. This phenomenon has been confirmed to some extent by studies using trichostatin A shown in Fig. 1 or trapoxin analogs shown in Fig. 2, which are enzyme inhibitors against histone deacetylase. In addition, when these inhibitors are allowed to act on cells at higher concentrations, cell cycle inhibition is caused and consequently growth inhibition occurs. Trichostatin A exhibits a non-competitive enzyme-inhibiting action

at low concentrations and is a reversible inhibitor; on the other hand, trapoxin analogs exhibit competitive inhibitory actions but are irreversible inhibitors. Further, it has also been reported that enzymatically active subunits of human histone deacetylase were purified on an affinity column using K-trap of a cyclic tetrapeptide compound similar to trapoxin; thus, strong evidence has been given to demonstrate that the cyclic tetrapeptide structure as found in trapoxin and the like forms a selective intermolecular linkage with said enzymatically active subunit.

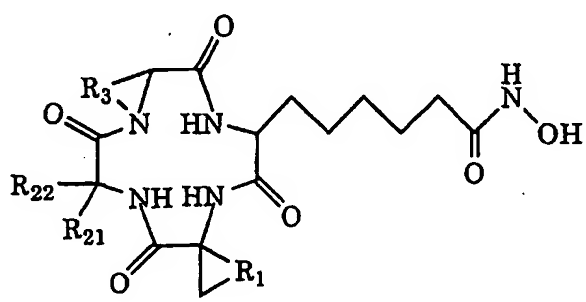
[0007] As stated above, since an enzyme inhibitor against histone deacetylase can be a drug causing cell differentiation or normal morphogenesis, it may also exhibit a promotion of the expression of MHC class-I molecule which occurs as a step in the process of differentiation; however, no report confirming this possibility has been made to date. Accordingly, there is a strong need for search and proposal of histone deacetylase enzyme inhibitors that exhibit promoting actions on the expression of MHC class-I molecule in self tissue cells. Further, as stated above, a histone deacetylase enzyme inhibitor at a high concentration causes the inhibition of cell cycle and consequently exhibits growth-inhibiting action, so a need exists for the proposal of a novel anti-cancer agent that is based on the promotion of the MHC class-I molecule expression and which exhibits a combined anti-cancer action due to the contributions of not only the inhibition of tumorization and the elimination of cancer cells by immune system, but also the cell growth-inhibiting action, all being associated with the promotion of MHC class-I molecule expression.

[0008] An object of the present invention is therefore to provide a novel histone deacetylase enzyme inhibitor exhibiting a promoting action on the expression of MHC class-I molecule in self tissue cells.

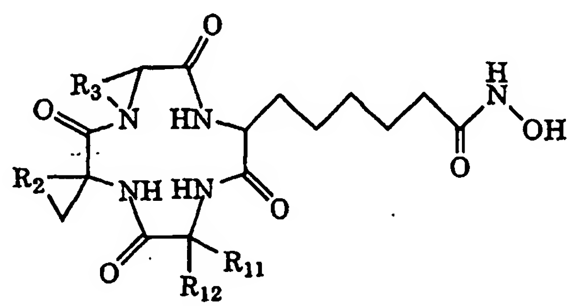
[0009] Another object of the present invention is to provide a pharmaceutical composition comprising said histone deacetylase enzyme inhibitor as an active ingredient.

DISCLOSURE OF THE INVENTION

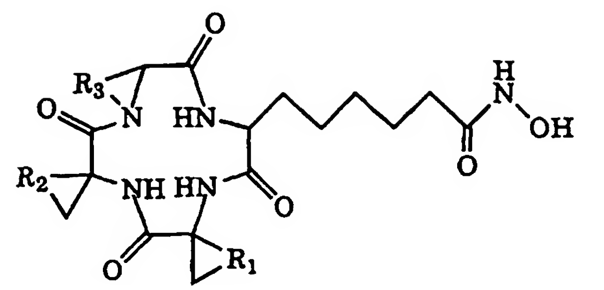
[0010] The present invention provides a cyclic tetrapeptide derivative represented by the following general formula (I), (I'), (I") or (I"') or a pharmaceutically acceptable salt thereof:



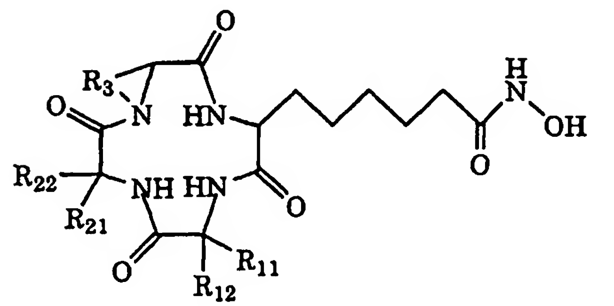
(I)



(I')



(I")



(I"')

wherein

each of R_{11}, R_{12}, R_{21} and R_{22} independently denotes hydrogen, a linear C_1-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached, or a branched C_3-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached; and
each of R_1, R_2 and R_3 independently denotes a linear C_1-C_5-alkylene group which may have a C_1-C_6 side chain, in which the side chain may form a condensed ring structure on the alkylene chain;
provided that at least one of R_{11}, R_{12}, R_{21} and R_{22} in general formula (I"') is a cyclohexyl methyl group.

[0011] The present invention also provides a histone deacetylase inhibitor and an MHC class-I molecule expression-promoting agent, each comprising the cyclic tetrapeptide derivative or pharmaceutically acceptable salt thereof as an active ingredient.
[0012] Further, the present invention provides a pharmaceutical composition comprising the cyclic tetrapeptide derivative or pharmaceutically acceptable salt thereof as an active ingredient. This pharmaceutical composition may be preferably used as an anti-cancer agent.
[0013] This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 11-53851, which is a priority document of the present application.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Fig. 1 shows the molecular structures of trichostatin A and trapoxin, as well as the action thereof to inhibit histone deacetylation.

Fig. 2 shows the molecular structures of trapoxin analogs.

BEST MODE FOR CARRYING OUT THE INVENTION

[0015] The cyclic tetrapeptide derivatives of the present invention and processes for preparing them will be herein-

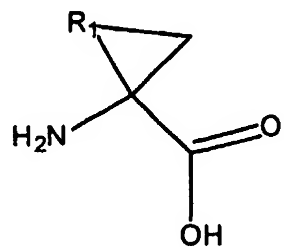
after described in more detail. In addition, the pharmacological activities of said cyclic tetrapeptide derivatives will be generally described.

[0016] As stated above, the cyclic tetrapeptide derivatives of the present invention are represented by any one of the four mutually related structures shown by general formulae (I) to (I'''). In general formulae (I) to (I'''), each of R_{11}, R_{12}, R_{21} and R_{22} independently denotes hydrogen, a linear C_1-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached, or a branched C_3-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached. Examples of a linear C_1-C_6-alkyl group and a branched C_3-C_6-alkyl group may include a methyl group, an ethyl group, a propyl group, a butyl group, a pentyl group, a hexyl group, an isopropyl group, an isobutyl group, a t-butyl group, a sec-pentyl group, a tert-pentyl group, a 1-ethyl-1-methylpropyl group, and a 1,1-dimethylpropyl group. Examples of a non-aromatic cycloalkyl group include a cyclopropyl group, a cyclobutyl group, a cyclopentyl group, and a cyclohexyl group. Examples of a linear or branched C_1-C_6-alkyl group to which an optionally substituted aromatic ring is attached include a benzyl group, a 4-methoxybenzyl group, a 3-indolylmethyl group, an (N-methoxy-3-indolyl)methyl group, and a 4-nitrobenzyl group.

[0017] In general formulae (I) to (I'''), each of R_1, R_2 and R_3 independently denotes a linear C_1-C_5-alkylene group which may have a C_1-C_6 side chain. Examples of a C_1-C_6 side chain include a methyl group, an ethyl group, a propyl group, a butyl group, a pentyl group, and a hexyl group. The side chain may form a condensed ring structure on the alkylene chain. Examples of an alkylene chain which forms such a condensed ring structure include a 1,2-phenylene group, a 1,3-phenylene group, a 2,3-pyridylene group, a 4,5-pyridinylene group, a 3,4-isoxazonylene group, a 2,4-pyridinylene group, a 3,4-pyridinylene group, a 2,3-pyrazinylene group, a 4,5-pyrimidinylene group, a 3,4-pyridazinylene group, and a 4,5-pyridazinylene group.

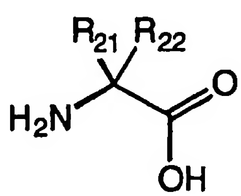
[0018] First, it will be described that these four molecular structures represented by general formulae (I) to (I''') have mutually close relationship to each other in their structures as stated below and that in this sense, they are compounds having a high degree of structural similarity.

[0019] The cyclic tetrapeptide derivative represented by general formula (I) according to the present invention is obtained by first linking the four constituent amino acids to prepare a corresponding chained tetrapeptide derivative and then cyclizing the chained tetrapeptide derivative. Thus, a cyclic tetrapeptide skeleton is formed through peptide linkages of the four α-amino acids represented by the following general formulae (II) to (V), i.e., an α-amino acid represented by general formula (II):



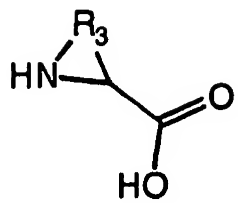
(II)

wherein R_1 denotes the same groups as R_1 in general formula (I); an α-amino acid represented by general formula (III):



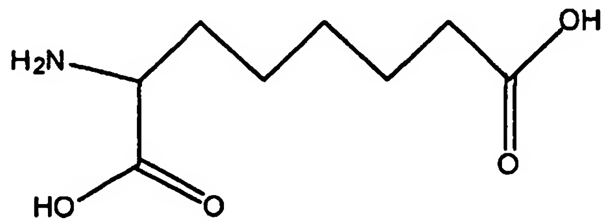
(III)

wherein R_{21} and R_{22} denote the same groups as R_{21} and R_{22} in general formula (I), respectively; a cyclic α-amino acid represented by general formula (IV):



(IV)

wherein R_3 denotes the same group as R_3 in general formula (I); and an α-amino acid represented by general formula (V):



(V),

and then a hydroxamic acid is derived from the side chain carboxyl group in the above general formula (V).

[0020] The cyclic tetrapeptide derivative represented by general formula (I') has an α-amino acid with a cyclic side chain containing an α-carbon atom at a different position from the cyclic tetrapeptide derivative of above general formula (I). Thus, the cyclic tetrapeptide derivative of general formula (I) has an α-amino acid with a cyclic side chain containing an α-carbon atom at a position where the amino acid of general formula (II) is found, while the cyclic tetrapeptide derivative of general formula (I') has an α-amino acid with a cyclic side chain containing an α-carbon atom at a position where the amino acid of general formula (III) is found in general formula (I).

[0021] Similarly, the cyclic tetrapeptide derivative represented by general formula (I") has α-amino acids, each having a cyclic side chain containing an α-carbon atom, at both positions where the amino acids of general formulae (II) and (III) are found in general formula (I).

[0022] Further, the derivative represented by general formula (I"') has no α-amino acid with a cyclic side chain containing an α-carbon atom at either position where the amino acid of general formula (II) or (III) is found in general formula (I).

[0023] In the cyclic peptide represented by general formula (I) of the present invention, the configuration of their constituent α-amino acids may be either L- or D-configuration; preferably, at least one amino acid residue has a different configuration than the other amino acid residues in order to ensure structural stability. Illustratively, at least one or two of the four α-amino acids advantageously has D-configuration while the remainder have L-configuration. When an α-amino acid with a ring structure containing an α-carbon atom has no branch, that is, it is optically inactive, the amino acid of either general formula (III) or (IV) may desirably take D-configuration.

[0024] More preferably, among the above four amino acids, D-configuration may be chosen for the cyclic amino acid represented by general formula (IV), while the remaining three take L-configuration; or D-configuration may be chosen for the amino acids represented by general formulae (II) and (IV), while the remaining two take L-configuration. It should also be noted that in the cyclic peptide of interest, a site close to the enzymatically active site of histone deacetylase is not the side chain of N-acetylated lysine which is an inherent substrate for the enzyme but hydroxamic acid derived from the side chain carboxyl group in the amino acid of general formula (V), so it is more preferable to select L-configuration for the amino acid of general formula (V) as in the case of naturally occurring lysine.

[0025] The remaining portion of the cyclic tetrapeptide plays such a role that the side chain hydroxamic acid structure derived from the amino acid of the above general formula (V) is directed to the enzymatically active site of histone deacetylase and held there. This role is substantially identical with the function of the cyclic peptide portion of trapoxin analogs which are known irreversible inhibitors. Thus, the remaining portion of the cyclic tetrapeptide provides intermolecular linkage to the neighborhood of the enzymatically active site of histone deacetylase, thereby ensuring that the side chain hydroxamic acid structure derived from the amino acid of the above general formula (V) is fixed onto the enzymatically active site.

[0026] Therefore, the remaining three α-amino acids may be of any types so long as their side chains are utilized in binding the peptide to the surface of the histone deacetylase protein. The cyclic amino acid of general formula (IV) is

a main functional part for fixing the direction of the side chain hydroxamic acid structure derived from the amino acid of the above general formula (V). The ring structure of the cyclic amino acid of general formula (IV) is preferably a 5-membered ring that is the same as the naturally occurring D-proline in trapoxin B shown in Fig. 2 or a 6-membered ring that is the same as the naturally occurring D-piperidine-2-carboxylic acid in trapoxin A shown in Fig. 2. Thus, the divalent group R_3 constituting this ring is preferably a trimethylene group in proline, a tetramethylene group in piperidine-2-carboxylic acid, or an unsaturated linear hydrocarbon group having a carbon-carbon double bond in correspondence to the linear hydrocarbon group with a chain length of 3 or 4. Alternatively, in these divalent hydrocarbon groups, one or more carbon atoms other than the carbon atom with the free valence which forms a bond to the amino nitrogen atom and the carbon atom at α-position of the carboxylic acid, may be replaced by any heteroatom such as oxygen, sulfur or nitrogen. Inter alia, R_3 is more preferably a trimethylene group or a tetramethylene group.

[0027] Generally, the remaining two α-amino acids preferably have a side chain as bulky as naturally occurring α-amino acids. That is to say, the side chain should not be more bulky than a p-hydroxybenzyl group of tyrosine, a benzyl group of phenylalanine or a 3-indolylmethyl group of tryptophan in the trapoxin analogs shown in Fig. 2. Particularly preferred examples include leucine, isoleucine, norleucine, cyclohexylalanine, and cyclic aminocarboxylic acids of various ring sizes.

[0028] The cyclic tetrapeptide derivative of general formula (I') according to the present invention differs from the cyclic tetrapeptide derivative of general formula (I) only in respect of the position of an α-amino acid with a ring structure containing an α-carbon atom. Therefore, the amino acids of general formulae (II) to (V) preferably selected for the cyclic tetrapeptide derivative of general formula (I) are also preferred for the cyclic tetrapeptide derivative of general formula (I'). With respect to the configuration of the amino acids of general formulae (II) to (V), it is also more preferred to select D-configuration for the cyclic α-amino acid of general formula (IV) and the α-amino acid of general formula (II) while the remaining two α-amino acids take L-configuration, and vice versa.

[0029] The cyclic tetrapeptide derivative of general formula (I") according to the present invention differs from the cyclic tetrapeptide derivatives of general formula (I) only in that it has an additional α-amino acid with a ring structure containing an α-carbon atom at a position where the amino acid of general formula (III) is found in general formula (I). Therefore, the amino acids of general formulae (II) to (IV) preferably selected for the cyclic tetrapeptide derivative of general formula (I) are also preferred for the cyclic tetrapeptide derivative of general formula (I").

[0030] The cyclic tetrapeptide derivative of general formula (I''') according to the present invention differs from the cyclic tetrapeptide derivative of general formula (I) only in that the amino acid of general formula (II) in general formula (I) is replaced by an α-amino acid without a ring structure containing an α-carbon atom. Therefore, the amino acid of general formula (III) preferably selected for the cyclic tetrapeptide derivative of general formula (I) is also preferred for the cyclic tetrapeptide derivative of general formula (I''').

[0031] As stated above, the cyclic tetrapeptide derivative of general formula (I) according to the present invention may be prepared by processes for the formation of peptide chain and cyclization using the four α-amino acids represented by general formulae (II) to (V) as the starting materials. One example of the processes will be generally described below.

<u>Process for Preparation</u>

[0032] The cyclic tetrapeptide derivative of the present invention may be prepared by first preparing a chained tetrapeptide intermediate in which the four α-amino acids represented by general formulae (II) to (V) are linked through peptide linkage, then converting it to a cyclic tetrapeptide, and eventually derivatizing the side chain carboxyl group of the α-amino acid represented by general formula (V) to a hydroxamic acid structure. Hereinbelow the process for the preparation will be generally described. The chained tetrapeptide intermediate may be used in a structure which is cleaved at any of the peptide linkages in the desired cyclic tetrapeptide derivative. In the description below, however, a synthesis route via a chained tetrapeptide intermediate having the cyclic α-amino acid represented by general formula (IV) at the C-terminal and the α-amino acid represented by general formula (V) at the N-terminal will be given as an example.

(Step 1) Synthesis of chained di-, tri- and tetrapeptides

[0033] First, according to a general procedure of peptide synthesis, amino acids of general formulae (III) and (IV) are linked to each other, an amino acid of general formula (II) is then linked, and finally an amino acid of general formula (V) whose side chain carboxyl group has been protected by benzyl esterification is linked to form a chained tetrapeptide.

[0034] In this process, Boc-group (t-butoxycarbonyl group) or Z-group (benzyloxycarbonyl group) is used to protect the amino groups of the starting amino acids, t-butyl ester is used to protect the carboxyl groups, and condensation is effected by DCC/HOBt method. The Z-group is removed by catalytic hydrogenolysis with Pd-C catalyst in acetic acid, which is then distilled off; and the free amine is extracted into ethyl acetate using aqueous sodium bicarbonate. An oily

product recovered from the extract is used in the subsequent condensation after vacuum drying.

[0035] The chained tetrapeptide entirely protected is purified by flash chromatography using a silica gel column.

(Step 2) Synthesis of cyclic peptide by very high dilution method

[0036] Using trifluoroacetic acid, the Boc-group and t-butyl ester in the chained tetrapeptide entirely protected are removed (deprotected). After distilling off trifluoroacetic acid from the reaction mixture, the product is precipitated with ether and petroleum ether and then vacuum dried.

[0037] One-tenth of the amount to be used of the peptide represented by general formula (VIII) is dissolved in DMF and adjusted to a concentration of 0.1 mM. To the DMF solution under ice cooling, a tertiary amine, e.g., diisopropylethylamine and HATU (O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) is added, and stirred at room temperature for 30 minutes. Subsequently, 1/10 of the amount to be used of the peptide represented by general formula (VIII) and diisopropylethylamine and HATU are added to the above DMF solution and stirred at room temperature for 30 minutes. These procedures were repeated 10 times in total to effect a cyclization reaction. After the reaction, the product (cyclic peptide) is extracted into ethyl acetate and then purified by flash chromatography using a silica gel column.

(Step 3) Introduction of side chain hydroxamic acid structure

[0038] The side chain benzyl ester of the cyclic peptide is removed by catalytic hydrogenolysis with Pd-C catalyst in methanol, and after distilling off methanol, vacuum drying was effected to yield a carboxylic acid as an oily product.

[0039] The cyclic peptide compound having a carboxylic acid on the side chain as obtained by the above deprotection and HOBt are dissolved in DMF, and under ice cooling, hydroxylamine hydrochloride, triethylamine and then BOP reagent are added and stirred for 1 hour. After the reaction, DMF was distilled off, decantation is performed with water, and then lyophilization is effected to yield a final product as a white powder. This white powder is dissolved in a small amount of methanol, purified using a semi-preparative column in HPLC, and lyophilized to yield a desired product represented by general formula (I).

[0040] The cyclic tetrapeptide derivatives represented by general formula (I'), (I") and (I"') according to the present invention may also be prepared by a similar procedure: first, a chained tetrapeptide is synthesized according to the above Step 1, then converted into a cyclic tetrapeptide utilizing the conditions of Step 2; and the side chain carboxyl group is converted into a side chain hydroxamic acid structure according to Step 3.

[0041] In addition to the above synthesis methods, the above compounds may also be synthesized by methods utilizing solid phase synthesis as illustrated in the below-mentioned Examples.

[0042] A pharmaceutically acceptable salt of the cyclic tetrapeptide derivative according to the present invention means, for example, a salt with a pharmaceutically acceptable inorganic acid, such as hydrochloride, and a salt with a pharmaceutically acceptable organic acid, such as acetate salt, if the derivative has basic nitrogen atoms.

[0043] The MHC class-I molecule expression-promoting agent of the present invention comprises as an active ingredient the cyclic tetrapeptide derivative having a hydroxamic acid structure (hydroxyaminocarbonyl structure) at the side chain terminal as described above, and the agent has an excellent expression-promoting activity as shown in the below-mentioned Test Examples. The MHC class-I molecule expression-promoting action is associated with histone deacetylase enzyme-inhibiting activity and this inhibition is considered to be reversible like trichostatin A having a hydroxamic acid structure. Further, advantages of high therapeutic effects are provided not only by cell growth inhibition and cell cycle-inhibiting action due to histone deacetylase enzyme inhibition which becomes remarkable upon administration at higher concentrations, but also by the complementary effect of action in eliminating cancer cells or cancer virus-infected cells associated with cytotoxic T cells due to promoted MHC class-I molecule expression. In addition, application as a drug is expected, which, when compared with trapoxin analogs which are irreversible inhibitors, allows unfavorable effects on the living body such as side-effects on normal tissue cells persist to a less degree and which, when compared with therapeutic effects, cause greatly reduced relative side-effects. Furthermore, by using the combination of non-aromatic α-amino acids presented herein to construct the cyclic tetrapeptide having a hydroxamic acid side chain, it is expected that the obtained compound will be resistant to attack by a metabolic enzyme, such as cytochrome P-450, and highly stable *in vivo*.

[0044] The pharmaceutical composition of the present invention attains therapeutic effects by utilizing the above MHC class-I molecule expression-promoting action; the dose of the cyclic tetrapeptide derivative as an active ingredient may be appropriately determined depending upon the object of the treatment, the degree of symptoms, the sex, age and body weight of a subject to be treated. When an adult male is to be treated, the daily dose is usually in the range of 0.1 to 50 mg/kg, preferably 0.5 to 10 mg/kg; this dose is preferably given in multiple sub-doses per day. The pharmaceutical composition may be formulated into any dosage form suitable for its administration route by adding an additive(s) generally used for peptide-like compound formulations of this type to the cyclic tetrapeptide derivative as

an active ingredient. Since the composition is high in cell permeability, a variety of administration routes can be used; dosage forms and administration routes commonly used to administer peptide hormones are preferred.

EXAMPLES

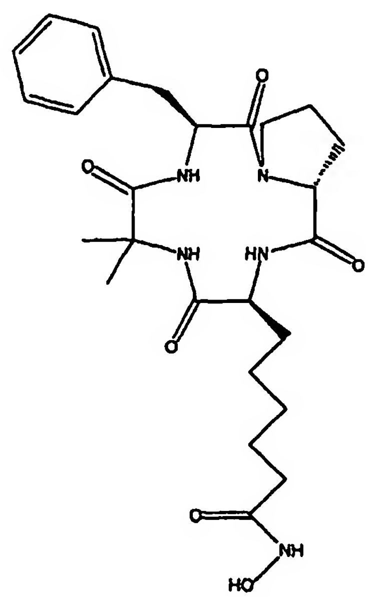
[0045] The cyclic tetrapeptide derivative of the present invention and processes for the preparation thereof as well as excellent physiological activities of said cyclic tetrapeptide derivative, i.e., excellent MHC class-I molecule expression-promoting action and histone deacetylase enzyme-inhibiting activity, will be described by way of examples.

[0046] In the following examples, abbreviations for non-naturally occurring amino acids mean the following amino acid residues:

Aib:	2-aminoisobutyric acid;
Asu(NHOH):	2-amino-8-hydroxamideoctanedioic acid;
Acc5:	1-aminocyclopentane-1-carboxylic acid;
Acc6:	1-aminocyclohexane-1-carboxylic acid;
Acc7:	1-aminocycloheptane-1-carboxylic acid;
Acc8:	1-aminocyclooctane-1-carboxylic acid;
1Ain:	1-aminoindane-1-carboxylic acid;
2Ain:	2-aminoindane-2-carboxylic acid;
Pip:	pipecolic acid;
Cha:	aminocyclohexylalanine.

[0047] Reference Example 1: Synthesis of CHAP-15; cyclo(-L-Asu(NHOH)-Aib-L-Phe-D-Pro-)

CHAP15



Step 1: Z-L-Phe-D-Pro-OtBu

[0048] Z-L-Phe-OH (2.25 g, 7.5 mmol) and H-D-Pro-OtBu (0.862 g, 5.0 mmol) were dissolved in DMF (10 ml), mixed with HOBt H_2O (766 mg, 5.0 mmol), BOP (3.3 g, 7.5 mmol) and triethylamine (1.75 ml, 12.5 mmol) under ice cooling, and then stirred for 2 hours. After the reaction mixture was concentrated, it was re-dissolved in ethyl acetate and washed sequentially with 10% citric acid, 4% $NaHCO_3$ and brine. After drying over anhydrous $MgSO_4$ and concentrating, the oily residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH = 99/1) to yield 1.403 g (62%) of the titled compound as an oil.
Rf = 0.83 ($CHCl_3$/MeOH = 9/1)

Step 2: H-L-Phe-D-Pro-OtBu

[0049] Z-L-Phe-D-Pro-OtBu (1.403 g, 3.1 mmol) was catalytically hydrogenated with Pd-C in acetic acid to remove the Z-group. After the reaction was continued for 10 hours, Pd-C was filtered off and acetic acid was concentrated. The residue was neutralized with 4% $NaHCO_3$ and extracted into ethyl acetate. The extract was dried over Na_2CO_3 and concentrated to yield 0.853 g (86%) of the titled compound.
Rf = 0.50 ($CHCl_3$/MeOH = 9/1)

Step 3: Z-Aib-L-Phe-D-Pro-OtBu

[0050] H-L-Phe-D-Pro-OtBu (0.853 g, 2.68 mmol) and Z-Aib-OH (954 mg, 4.02 mmol) were condensed as described in Step 1 above. The oily product was purified by flash chromatography on silica gel ($CHCl_3$/MeOH = 99/1) to yield 1.23 g (85%) of the titled compound.
Rf = 0.75 ($CHCl_3$/MeOH = 9/1)

Step 4: H-Aib-L-Phe-D-Pro-OtBu

[0051] Z-Aib-L-Phe-D-Pro-OtBu (1.23 g, 2.28 mmol) was catalytically hydrogenated with Pd-C in acetic acid to remove the Z-group. After the reaction was continued for 10 hours, Pd-C was filtered off and acetic acid was concentrated. The residue was neutralized with 4% $NaHCO_3$ and extracted into ethyl acetate. The extract was dried over Na_2CO_3 and concentrated to yield 0.726 g (1.80 mmol; 79%) of the titled compound.
Rf = 0.50 ($CHCl_3$/MeOH = 9/1)

Step 5: Boc-L-Asu(OBzl)-Aib-L-Phe-D-Pro-OtBu

[0052] H-Aib-L-Phe-D-Pro-OtBu (0.726 g, 1.80 mmol) and Boc-L-Asu(OBzl)-OH (1.02 g, 2.70 mmol) were condensed as described in Step 1 above. The oily product was purified by flash chromatography on silica gel ($CHCl_3$/MeOH = 49/1) to yield 993 mg (72%) of the titled compound.
Rf = 0.71 ($CHCl_3$/MeOH = 9/1)

Step 6: H-L-Asu(OBzl)-Aib-L-Phe-D-Pro-OH.TFA

[0053] Trifluoroacetic acid (3 ml) was added to Boc-L-Asu(OBzl)-Aib-L-Phe-D-Pro-OtBu (993 mg, 1.30 mmol) under ice cooling to remove the Boc-group and t-butyl ester at room temperature for 2 hours. After distilling off trifluoroacetic acid, the residue was precipitated with ether/petroleum ether (1:5) and then vacuum dried to yield 737 mg (78%) of the titled compound.
HPLC: Rt = 8.66 min (column: Wako pak C4, 4.6 × 150 mm, 30-100% linear gradient CH_3CN/0.1% TFA over 30min, flow rate 1.0 ml/min)

Step 7: Cyclo(-L-Asu(OBzl)-Aib-L-Phe-D-Pro-)

[0054] H-L-Asu(OBzl)-Aib-L-Phe-D-Pro-OH TFA (30 mg, 0.042 mmol) was dissolved in DMF (400 ml) and adjusted to a concentration of 0.1 mM. To the DMF solution, 10% DIEA/DMF (0.3 ml, 0.17 mmol) and HATU (25 mg, 0.066 mmol) were added and stirred at room temperature for 30 minutes. This procedure was repeated 10 times in total to perform a cyclization reaction. After the reaction, the reaction mixture was concentrated, dissolved in ethyl acetate, and washed sequentially with 10% citric acid, 4% $NaHCO_3$ and brine. After drying over anhydrous $MgSO_4$ and concentrating, the oily residue was purified by flash chromatography on silica gel ($CHCl_3$/MeOH = 99/1) to yield 151 mg (61%) of the titled compound.
Rf = 0.78 ($CHCl_3$/MeOH = 9/1)
HPLC: Rt =18.15 min (column: MS GEL C18, 4.6 × 150 mm 30-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)

Step 8: Cyclo(-L-Asu-Aib-L-Phe-D-Pro-)

[0055] Cyclo(-L-Asu(OBzl)-Aib-L-Phe-D-Pro-)(151 mg, 0.256 mmol) was dissolved in methanol (5 ml) and catalytically hydrogenated with Pd-C to remove the benzyl ester group. After the reaction was continued for 5 hours, Pd-C was filtered off and methanol was concentrated to yield 124 mg (97%) of the titled compound.
HPLC: Rt = 6.32 min (column: Wako pak C4, 4.6 × 150 mm, 30-100% linear gradient CH_3CN/0.1% TFA over 30 min,

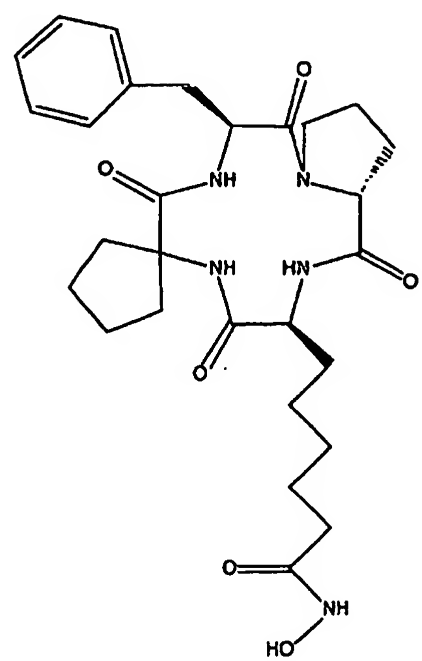
flow rate 1.0 ml/min)

Step 9: Cyclo(-L-Asu(NHOH)-Aib-L-Phe-D-Pro-)

[0056] Cyclo(-L-Asu-Aib-L-Phe-D-Pro-)(124 mg, 0.248 mmol) was dissolved in DMF (3 ml), and then mixed with hydroxylamine hydrochloride (86 mg, 1.24 mmol), HOBt H_2O (57 mg, 0.372 mmol) and BOP (165 mg, 0.372 mmol) under ice cooling. Triethylamine (0.24 ml, 1.74 mmol) was added and stirred for 2 hours. The reaction mixture was concentrated to 1 ml, and then purified by elution from a LH-20 column (2 × 85 cm) with DMF. The eluate was lyophilized to yield 95 mg (74%) of the titled compound.
HPLC: Rt =16.32 min (column: Wako pak C18, 4.6 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 516 (M+H)+

Example 1: Synthesis of CHAP-54; cyclo(-L-Asu(NHOH)-Acc5-L-Phe-D-Pro-)

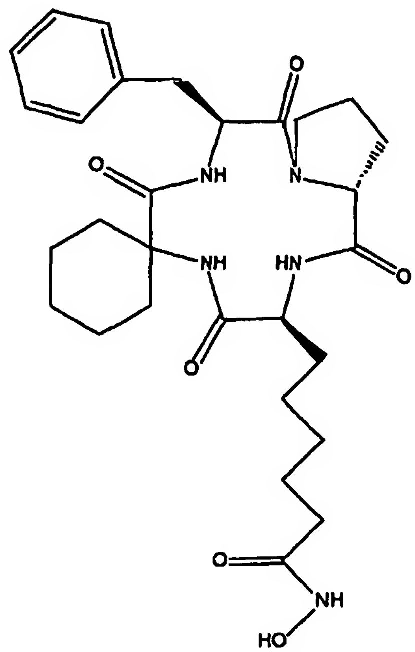
[0057]



[0058] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt = 17.04 min (column: MS GEL C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 542 (M+H)+

Example 2: Synthesis of CHAP-55; cyclo(-L-Asu(NHOH)-Acc6-L-Phe-D-Pro-)

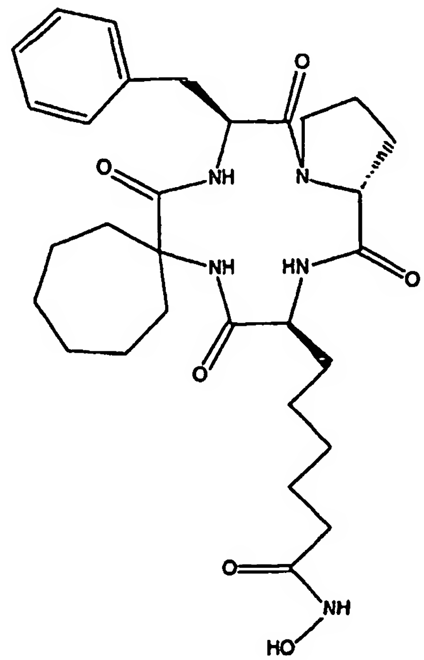
[0059]



[0060] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt = 18.25 min (column: MS GEL C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 556 (M+H)+

Example 3: Synthesis of CHAP-71; cyclo(-L-Asu(NHOH)-Acc7-L-Phe-D-Pro-)

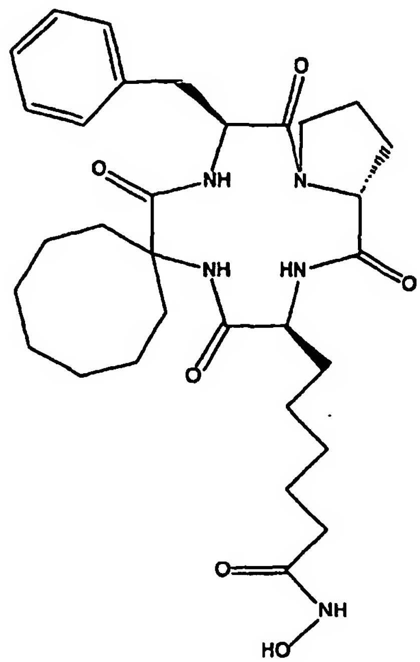
[0061]



[0062] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt =19.24 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 570 (M+H)+

Example 4: Synthesis of CHAP-76; cyclo(-L-Asu(NHOH)-Acc8-L-Phe-D-Pro-)

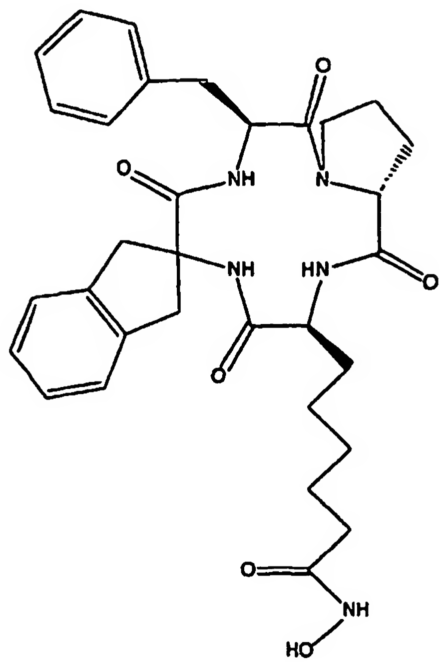
[0063]



[0064] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt = 18.72 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 584 (M+H)+

Example 5: Synthesis of CHAP-81; cyclo(-L-Asu(NHOH)-2Ain-L-Phe-D-Pro-)

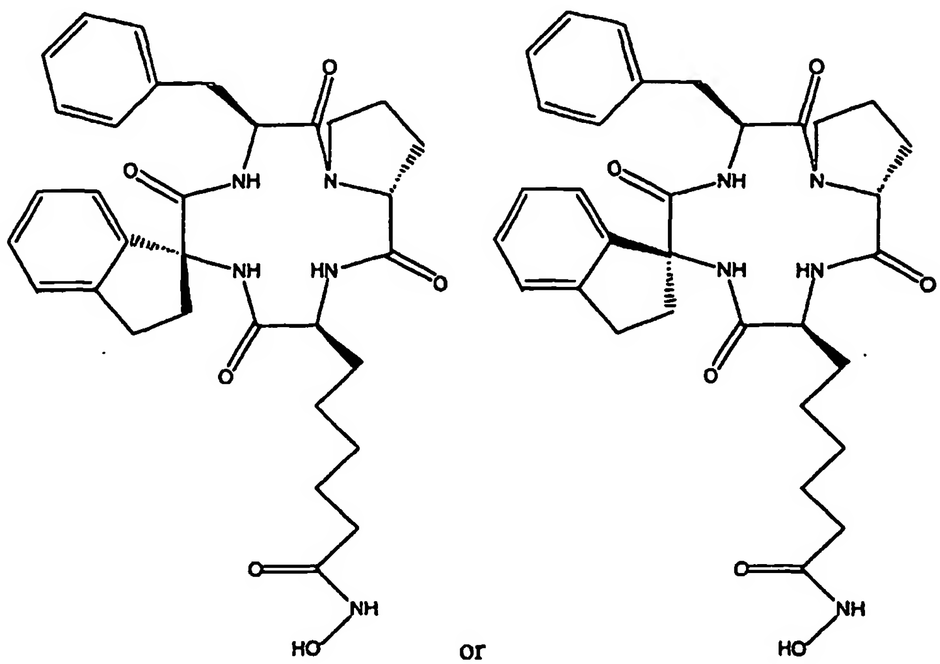
[0065]



[0066] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt = 18.20 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/ z= 590 (M+H)+

Example 6: Synthesis of CHAP-82; cyclo(-L-Asu(NHOH)-1 Ain(f)-L-Phe-D-Pro-)

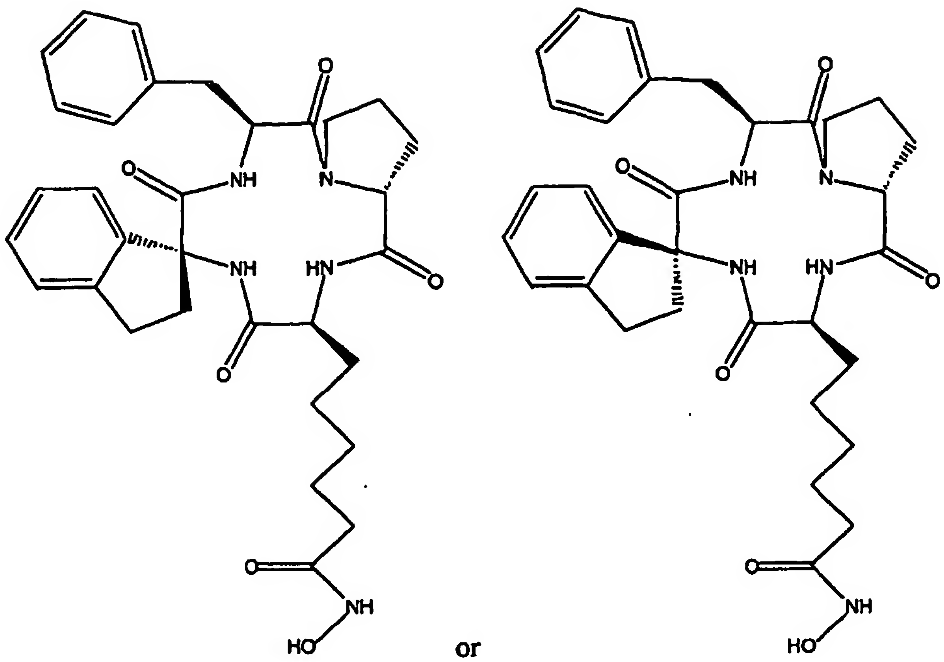
[0067]



[0068] This compound was synthesized in accordance with the synthesis method for CHAP-15. 1Ain(f) is 1-aminoindane carboxylic acid as in the case of 1Ain(s) in Example 7. The former is eluted faster than the latter in HPLC, indicating that they are a pair of diastereomers. However, their configurations have not been determined.
HPLC: Rt = 16.26 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 590 (M+H)+

Example 7: Synthesis of CHAP-83; cyclo(-L-Asu(NHOH)-1Ain(s)-L-Phe-D-Pro-)

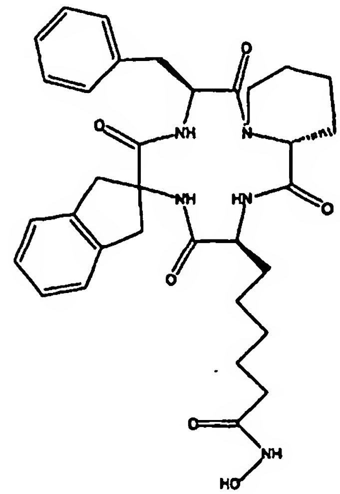
[0069]



[0070] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt = 17.13 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 590 (M+H)+

Example 8: Synthesis of CHAP-91; cyclo(-L-Asu(NHOH)-2Ain-L-Phe-D-Pip-)

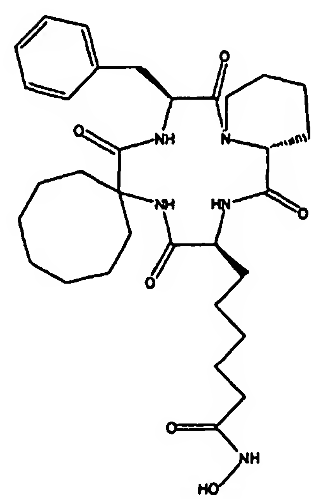
[0071]



[0072] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt = 19.07 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 604 (M+H)+

Example 9: Synthesis of CHAP-90; cyclo(-L-Asu(NHOH)-Acc8-L-Phe-D-Pip-)

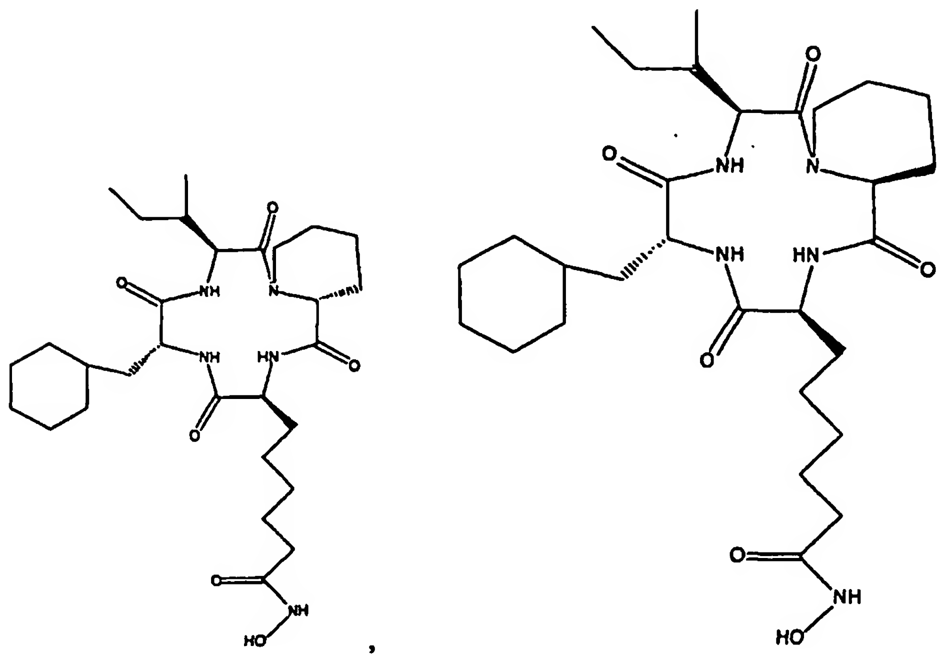
[0073]



[0074] This compound was synthesized in accordance with the synthesis method for CHAP-15.
HPLC: Rt =20.08 min (column: Wako pak C18, 4.6 × 150 mm, 0-100% linear gradient CH_3CN/0.1% TFA over 30 min, flow rate 1.0 ml/min)
FAB-MS: m/z = 598 (M+H)+

Example 10: Synthesis of CHAP-86; cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-D-Pip-) and CHAP-87; cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-L-Pip-)

[0075]



CHAP86 CHAP87

Step 1: Boc-L-Ile-D,L-Pip-OBzl

[0076] Boc-L-Ile-OH 1/2 H_2O (3.92 g, 16.8 mmol), H-D,L-Pip-OBzl HCl (3.57 g, 12 mmol) and HATU (7.75 g, 18 mmol) were dissolved in DMF (15 ml), mixed with Et_3N (4.76 ml, 30 mmol) under ice cooling, and then stirred overnight. The reaction mixture was concentrated, extracted with ethyl acetate (120 ml), and washed twice with 10% citric acid, 4% $NaHCO_3$ and then brine, respectively. After drying over magnesium sulfate, ethyl acetate was distilled off. The residue was vacuum dried and purified by flash chromatography (3.5 × 20 cm) with $CHCl_3$/MeOH (99:1) to yield Boc-L-Ile-D,L-Pip-OBzl (5.90 g, 13.8 mmol; yield 97%) as an oil.
Rf = 0.50 ($CHCl_3$/MeOH = 49/1)

Step 2: Boc-D-Cha-L-Ile-D,L-Pip-OBzl

[0077] Boc-L-Ile-D,L-Pip-OBzl (432 mg, 1 mmol) was dissolved in TFA (3 ml) and allowed to stand for 30 minutes under ice cooling. The reaction solution was distilled and dried under reduced pressure to yield H-L-Ile-D,L-Pip-OBzl TFA (638 mg; yield 144%), which was then dissolved in DMF (5 ml). The solution was mixed with Boc-D-Cha-OH (326 mg, 1.2 mmol) and subsequently mixed with HOBt H_2O (230 mg, 1.5 mmol, 1.5 equivalents), Et_3N (0.52 ml, 3.7 mmol, 3.7 equivalents) and BOP (663 mg, 1.5 mmol, 1.5 equivalents) under ice cooling, followed by stirring at room temperature for 15 hours. The reaction mixture was concentrated, extracted with ethyl acetate (100 ml), and washed twice with 10% citric acid, 4% $NaHCO_3$ and brine, respectively. After drying over magnesium sulfate, ethyl acetate was distilled off. The residue was vacuum dried and purified by flash chromatography (2.4 × 14 cm) with $CHCl_3$/MeOH (99:1) to yield Boc-D-Cha-L-Ile-D,L-Pip-OBzl (565 mg, 0.96 mmol; yield 96%) as a foam.
Rf = 0.27 ($CHCl_3$/MeOH = 49/1)

Step 3: Boc-D-Cha-L-Ile-D,L-Pip-OH

[0078] Boc-D-Cha-L-Ile-D,L-Pip-OBzl (565 mg, 0.96 mmol) was dissolved in MeOH (10 ml). Pd-C catalyst (250 mg) was added to the solution and the solution was stirred for 15 hours under hydrogen atmosphere to remove the benzyl ester group. After Pd-C catalyst was filtered off, MeOH was distilled off to give a residue, which was then vacuum dried to yield Boc-D-Cha-L-Ile-D,L-Pip-OH (459 mg, 0.93 mmol; yield 97%) as a foam.

Step 4: Boc-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OTmse

[0079] Boc-L-Asu(OBzl)-OTmse (535 mg, 1.1 mmol, 1.2 equivalents) was mixed with TFA (3 ml) under ice cooling and allowed to stand at room temperature for 30 minutes to remove the Boc-group. After distilling off TFA, the reaction mixture was dried under reduced pressure to yield H-L-Asu(OBzl)-OTmse TFA as an oil, which was then dissolved in DMF (5 ml). The solution was mixed with Boc-D-Cha-L-Ile-D,L-Pip-OH (459 mg, 0.93 mmol) and subsequently mixed with HOBt H_2O (213 mg, 1.4 mmol, 1.5 equivalents), BOP (616 mg, 1.4 mmol, 1.5 equivalents) and Et_3N (0.48 ml, 3.5 mmol, 3.7 equivalents) under ice cooling, followed by stirring at room temperature for 15 hours. The reaction mixture was concentrated, extracted with ethyl acetate (100 ml), and washed twice with 10% citric acid, 4% $NaHCO_3$ and brine, respectively. After drying over magnesium sulfate, ethyl acetate was distilled off. The residue was vacuum dried and purified by flash chromatography (2.4 × 20 cm) with $CHCl_3$/MeOH (99:1) to yield Boc-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl) -OTmse (813 mg, 0.93 mmol; yeild 100%) as an oil.
Rf = 0.20 ($CHCl_3$/MeOH = 49/1)

Step 5: H-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OH TFA

[0080] Boc-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OTmse (813 mg, 0.95 mmol) was dissolved in DMF (2 ml), mixed with a 1M THF solution of tetrabutylammonium fluoride (4 ml, 4 mmol, 4 equivalents), and then allowed to stand at room temperature for 30 minutes. The reaction mixture was concentrated, acidified by addition of 10% citric acid, and then extracted with ethyl acetate (100 ml). The ethyl acetate phase was washed twice with brine, dried over magnesium sulfate, and then distilled to remove ethyl acetate. The residue was vacuum dried to yield Boc-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OH (696 mg, 0.91 mmol; yeild 97%). Boc-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OH (696 mg, 0.91 mmol) was mixed with TFA (5 ml) under ice cooling and then allowed to stand at room temperature for 30 minutes to remove the Boc-group. After distilling off TFA, the residue was precipitated with ether/petroleum ether (1:10), filtered and dried under reduced pressure to yield H-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OH TFA (752 mg, 0.91 mmol; yield 100%).

Step 6: Cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-D,L-Pip-)

[0081] H-D-Cha-L-Ile-D,L-Pip-L-Asu(OBzl)-OH TFA (752 mg, 0.91 mmol) was dissolved in DMF (5 ml), 1 ml of which was then transferred to DMF (455ml, 0.4 mM) and mixed with 1 ml of a solution of HATU (104 mg, 1.5 equivalents) and DIEA (0.63 ml, 4 equivalents) in DMF (10 ml), followed by stirring at room temperature for 1 hour. The same procedure was repeated 5 times to effect a cyclization reaction. The reaction mixture was concentrated, extracted with ethyl acetate (100 ml), and then washed twice with 10% citric acid, 4% $NaHCO_3$ and brine, respectively. After drying over magnesium sulfate, ethyl acetate was distilled off. The residue was vacuum dried and purified by flash chromatography (2.4 × 40 cm) with $CHCl_3$/MeOH (99:1) to yield cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-D-Pip-)(90 mg, 0.14 mmol; yield 18%) and cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-D,L-Pip-)(200 mg, 0.31 mmol; yield 40%).

<u>cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-D-Pip-)</u>

[0082] Rf = 0.45 ($CHCl_3$/MeOH = 9/1)
HPLC: Rt = 19.40 min (column: Wakosil 5C4, 4.5 × 150 mm, 37-100% linear gradient CH_3CN/0.1% TFA over 30 min)

<u>cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-L-Pip-)</u>

[0083] Rf = 0.45 ($CHCl_3$/MeOH = 9/1)
HPLC: Rt = 16.80 min (column: Wakosil 5C4, 4.5 × 150 mm, 37-100% linear gradient CH_3CN/0.1% TFA over 30 min)

Step 7: Cyclo(-L-Asu(OH)-D-Cha-L-Ile-D-Pip-) and cyclo(-L-Asu(OH)-D-Cha-L-Ile-D,L-Pip-)

[0084] Cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-D-Pip-)(90 mg, 0.14 mmol) and cyclo(-L-Asu(OBzl)-D-Cha-L-Ile-D,L-Pip-) (200 mg, 0.31 mmol) were dissolved in MeOH (5 ml) and MeOH/DMF (10:1), respectively. Pd-C catalyst (250 mg) was

added to each solution and stirred for 15 hours under a stream of hydrogen to remove the benzyl ester group. After Pd-C catalyst was filtered off, acetic acid was distilled off to give a residue, which was then vacuum dried to yield cyclo (-L-Asu(OH)-D-Cha-L-Ile-D-Pip-)(76 mg, 0.14 mmol; yield 85%) and cyclo(-L-Asu(OH)-D-Cha-L-Ile-D,L-Pip-)(170 mg, 0.31 mmol; yield 99%).

cyclo(-L-Asu(OH)-D-Cha-L-Ile-D-Pip-)

[0085] HPLC: Rt = 12.64 min (column: Wakosil 5C4, 4.5 × 150 mm, 37-100% linear gradient CH_3CN/0.1% TFA over 30 min)

cyclo(-L-Asu(OH)-D-Cha-L-Ile-L-Pip-)

[0086] HPLC: Rt = 9.48 min (column: Wakosil 5C4, 4.5 × 150 mm, 37-100% linear gradient CH_3CN/0.1% TFA over 30 min)

Step 8: Cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-D-Pip-) and cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-D,L-Pip-)

[0087] Cyclo(-L-Asu(OH)-D-Cha-L-Ile-D-Pip-)(76 mg, 0.14 mmol) and cyclo(-L-Asu(OH)-D-Cha-L-Ile-D,L-Pip-)(170 mg, 0.31 mmol) were dissolved in DMF (3 ml), respectively. Each solution was mixed with HOBt H_2O (LDLD compound: 32 mg, 0.21 mmol, 1.5 equivalents; LDLD/LDLL mixture: 71 mg, 0.47 mmol, 1.5 equivalents) and NH_2OH HCl (LDLD compound: 49 mg, 0.70 mmol, 5 equivalents; LDLD/LDLL mixture: 108 mg, 1.6 mmol, 5 equivalents), and subsequently mixed with BOP (LDLD compound: 93 mg, 0.21 mmol, 1.5 equivalents; LDLD/LDLL mixture: 206 mg, 0.47 mmol, 1.5 equivalents) and Et_3N (LDLD compound: 0.12 ml, 0.84 mmol, 6 equivalents; LDLD/LDLL mixture: 0.20 ml, 1.86 mmol, 6 equivalents) under ice cooling, followed by stirring for 4 hours. After the reaction was completed, the generated Et_3N HCl was filtered off and DMF was distilled off. The LDLD compound was purified using a Sephadex LH-20 gel filtration column (2.4 × 85 cm, DMF), followed by lyophilization, to yield cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-D-Pip-)(20 mg, 0.035 mmol). The LDLD/LDLL mixture was dissolved in a small amount of MeOH and purified by HPLC (column: YMC-Pack C8 10 × 250 mm, 37% CH_3CN/0.1% TFA), followed by lyophilization, to yield cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-D-Pip-)(27 mg, 0.047 mmol; purity 100%) and cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-L-Pip-)(51 mg, 0.090 mmol; purity 93%).

cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-D-Pip-)

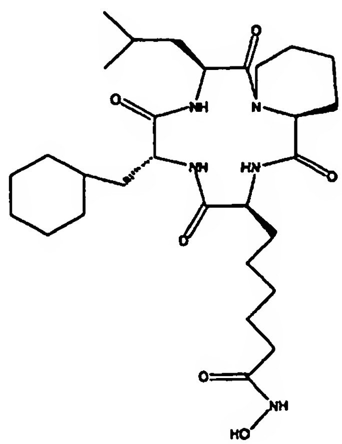
[0088] HPLC: Rt = 21.34 min (column: Wakosil II 5C18, 4.5 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min)

cyclo(-L-Asu(NHOH)-D-Cha-L-Ile-L-Pip-)

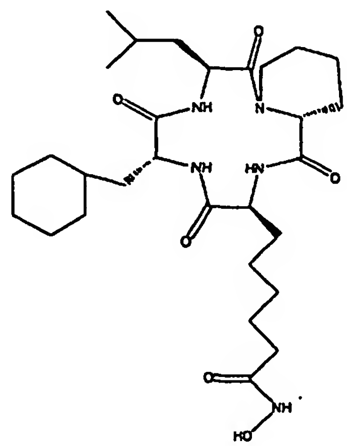
[0089] HPLC: Rt = 18.79 min (column: Wakosil II 5C18, 4.5 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min)
FAB-MS (2,2'-dithiodiethanol): m/z = 564 [M+H]+

Example 11: Synthesis of CHAP-84; cyclo(-L-Asu(NHOH)-D-Cha-L-Leu-L-Pip-) and CHAP-85; cyclo(-L-Asu(NHOH)-D-Cha-L-Leu-D-Pip-)

[0090]



CHAP84



CHAP85

[0091] The titled cyclic tetrapeptides were synthesized as described in Example 10 (CHAP86, CHAP87), except that BOP reagent (1.5 equivalents) and HOBt H_2O (1.5 equivalents) were used for the synthesis of Boc-L-Leu-D,L-Pip-OBzl.

cyclo(-L-Asu(NHOH)-D-Cha-L-Leu-D-Pip-)

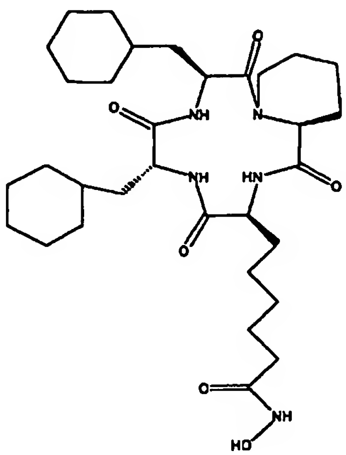
[0092] HPLC: Rt = 20.89 min (column: Wakosil II 5C18, 4.5 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min)

cyclo(-L-Asu(NHOH)-D-Cha-L-Leu-L-Pip-)

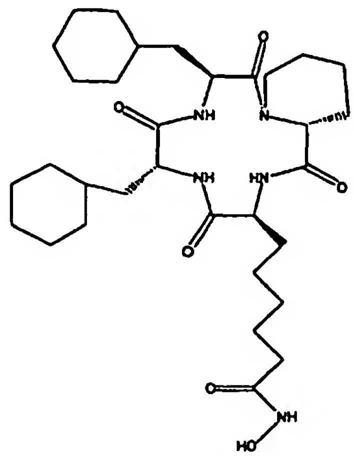
[0093] HPLC: Rt = 18.33 min (column: Wakosil II 5C18, 4.5 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min)
FAB-MS (2,2'-dithiodiethanol): m/z = 564 [M+H] +

Example 12: Synthesis of CHAP-78; cyclo(-L-Asu(NHOH)-D-Cha-L-Cha-L-Pip-) and CHAP-79; cyclo(-L-Asu(NHOH)-D-Cha-L-Cha-D-Pip-)

[0094]



CHAP78



CHAP79

[0095] The titled cyclic tetrapeptides were synthesized as described in Example 10 (CHAP86, CHAP87), except that DCC (1.3 equivalents) and HOBt H_2O (1.3 equivalents) were used for the synthesis of Boc-L-Cha-D,L-Pip-OBzl.

cyclo(-L-Asu(NHOH)-D-Cha-L-Cha-D-Pip-)

[0096] HPLC: Rt = 26.20 min (column: Wakosil II 5C18, 4.5 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min)

cyclo(-L-Asu(NHOH)-D-Cha-L-Cha-L-Pip-)

[0097] HPLC: Rt = 23.36 min (column: Wakosil II 5C18, 4.5 × 150 mm, 10-100% linear gradient CH_3CN/0.1% TFA over 30 min)
FAB-MS (2,2'-dithiodiethanol): m/z = 604 [M+H] +

Test Example 1: MHC class-I molecule expression-promoting activity

[0098] The cyclic tetrapeptide derivatives of the present invention were investigated for their MHC class-I molecule expression-promoting actions in the following test. Thus, in this test, the cyclic tetrapeptide derivatives of the present invention were allowed to act on cancer cells in order to demonstrated that they promote MHC class-I molecule expression.

Test Method:

[0099] The cancer cells used were mouse melanoma, B16/BL6 cells, provided by the National Cancer Institute, U. S.A. Said cells were cultured in MEM media supplemented with 10% FBS at 37 °C, 5% CO_2 in an humidified incubator.
[0100] A compound to be tested was preliminarily dissolved in dimethylsulfoxide (DMSO) and adjusted to a concentration of 100 mM or 10 mM (stock solution). A commercially available trichostatin A (purchased from Wako Pure Chemical, Japan) which had been proved to have a histone deacetylase enzyme-inhibiting activity, was also dissolved in DMSO and adjusted to a concentration of 5 mg/ml (16.54 mM)(stock solution). Trichostatin A was used as a positive control compound for MHC class-I molecule expression-promoting action due to histone deacetylase enzyme-inhibiting activity. DMSO used as a solvent for the stock solution of a test compound would inevitably be introduced into the medium in the test; however, it had been separately confirmed not to affect the test results in amounts within the range used in the test.

[0101] Said B16/BL6 cells were inoculated on a 96-well microplate at a cell density of 5000 cells per well, each well containing 200 μl of said medium. After culturing for 24 hours, 10μl of a sample containing a given amount of the stock solution of a test compound which had been diluted in the medium was added and cultured for additional 72 hours. Thereafter, each well was washed once with PBS (phosphate buffered saline) and floating cells and the medium were removed. Then, the well was treated with 0.1% glutaraldehyde solution for 3 minutes to fix the cells.

[0102] The amount of MHC class-I molecule expressed on the surface of the fixed cells was measured as follows. Anti-H-2$K^bD^bD^d$ antibody, which is an antibody against mouse MHC class-I molecule (commercially available from Meiji Milk Co., Ltd), was used as a primary antibody, biotinylated anti-mouse IgG+M (commercially available from Chemicon) was used as a secondary antibody, and streptavidin-β-galactosidase conjugate (commercially available from BRL) was reacted as a labeling enzyme. The amount of the thus labelled enzyme β-galactosidase was measured in a microplate reader by recording the fluorescent intensity (excitation: 365 nm, fluorescence: 450 nm) resulting from the enzyme reaction product using 4-methylumbelliferyl- β-D-galactoside (commercially available from Nacalai Tesque) as a substrate. A fluorescence intensity measured for another well to which no test compound was added and which was treated in a similar manner without adding said primary antibody was used as a background level. The value obtained by subtracting said background level from the actually measured value (an apparent value including the background level) was defined as a true measurement reflecting the amount of expressed MHC class-I molecule.

[0103] A group without the addition of any test compound was used as a control group and the measurement of MHC class-I molecule expressed in said group was used as a standard value. The amount of MHC class-I molecule expressed at an addition concentration of each test compound was shown as a relative amount based on said standard value set to one (1). The strength of each test compound activity was compared at a concentration which provides twice-promoted expression ($C_{\times 2}$).

[0104] Part of the results for the cyclic tetrapeptide derivatives of the present invention is shown in Table 1. All the cyclic tetrapeptides shown in Table 1 were demonstrated to have an excellent MHC class-I molecule expression-promoting activity.

Table 1

Test compound	Twice promoting concentration $C_{\times 2}$
CHAP15	33.2 nM
CHAP54	5.67 nM
CHAP55	5.38 nM
CHAP71	2.76 nM
CHAP76	1.88 nM
CHAP78	2.35 nM
CHAP79	2.79 nM
CHAP81	1.29 nM
CHAP82	2.69 nM
CHAP83	2.29 nM
CHAP84	5.65 nM
CHAP85	2.26 nM
CHAP86	3.29 nM
CHAP87	5.39 nM
CHAP90	3.96 nM
CHAP91	2.48 nM

Test Example 2: Inhibitory activity against histone deacetylase enzyme

[0105] In order to prove that the effects on the cell line in the above Test Example 1 are actually based on the inhibition of histone deacetylase by the cyclic tetrapeptide compounds of the present invention, inhibitory activity against histone deacetylase was examined in an *in vitro* system as follows.

Test Method:

[0106] The preparation of histone deacetylase was performed essentially according to the method of Yoshida et al. (J. Biol. Chem., 265, 17174-17179, 1990). The enzyme to be used was partially purified from B16/BL6 cells. The cells were suspended in HDA buffer (15 mM potassium phosphate, 5% glycerol, 0.2 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.5), homogenized and centrifuged (2500 × g, 10 min) to collect the nuclei, which were then homogenized in the same buffer containing 1 M $(NH_4)_2SO_4$. After ultrasonic disruption and centrifugation, the concentration of $(NH_4)_2SO_4$ in the collected supernatant was increased to 3.5 M to precipitate histone deacetylase. This precipitate was re-dissolved in HDA buffer, subjected to gel filtration to replace the solvent by HDA buffer, and used as a crude histone deacetylase enzyme solution.

[0107] As a substrate, a synthetic substrate peptide, [^{3}H]acetylated histone H4 peptide was used. This [^{3}H]acetylated histone H4 peptide was obtained by synthesizing the N-terminal peptide of histone H4; SGRGKGGKGLGKGGAKRHRKVC (the C-terminal being cysteine) and then radioactively acetylaing with ^{3}H-acetic anhydride.

[0108] An assay was performed by incubating the synthetic substrate solution and the enzyme solution at 37°C for 3 hours in the presence of a compound to be tested (reaction volume, 100 µl). The reaction was stopped by adding 25 µl of 1 M HCl and 0.2 M acetic acid, and [^{3}H]acetate cleaved by the enzyme reaction was extracted with ethyl acetate for radioactivity measurement. For a control group, the same procedure was repeated without addition of any test compound to the reaction system. Each test compound was evaluated for a concentration required to cause 50% inhibition of the histone deacetylase enzyme activity in the control group (50% inhibitory concentration).

[0109] Part of the results is shown in Table 2. All the cyclic tetrapeptides shown in Table 2 were demonstrated to have an excellent inhibitory activity against histone deacetylase.

Table 2

Test compound	50% inhibitory concentration
CHAP15	5.17 nM
CHAP54	3.02 nM
CHAP55	2.19 nM
CHAP71	2.14 nM
CHAP76	3.99 nM
CHAP78	3.88 nM
CHAP79	3.90 nM
CHAP81	0.980 nM
CHAP82	1.12 nM
CHAP83	1.95 nM
CHAP84	2.22 nM
CHAP85	1.96 nM
CHAP86	3.14 nM
CHAP87	1.98 nM
CHAP90	2.75 nM
CHAP91	1.20 nM

[0110] All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

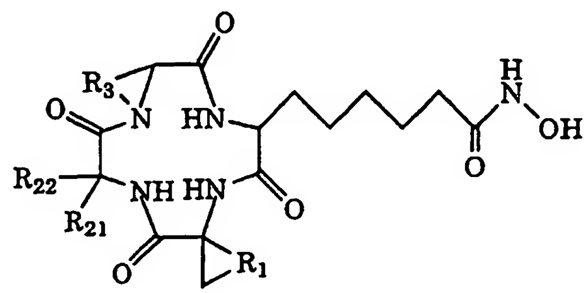
INDUSTRIAL APPLICABILITY

[0111] The cyclic tetrapeptide derivatives or pharmaceutically acceptable salts thereof according to the present invention have an excellent activity in promoting the MHC class-I molecule expression in association with their excellent inhibitory activity against histone deacetylase enzyme. Further, they also show inhibitory effects on cell proliferation and cell cycle, which are derived from the histone deacetylase inhibition, so that enlargement of cancer tissues is

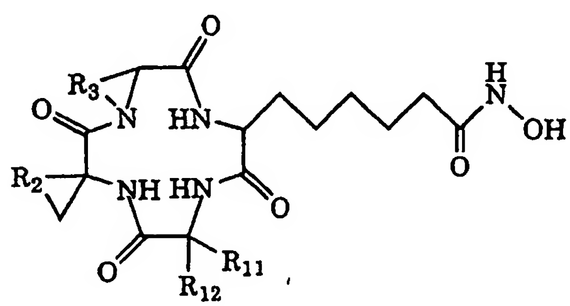
inhibited. Hence, by utilizing the MHC class-I molecule expression-promoting action, they can remarkably promote the elimination of cancer cells by the immune system and are very useful as anti-cancer agents. Since the histone deacetylase inhibition by the cyclic tetrapeptide derivatives of the present invention is reversible, they have the advantage of causing very little unfavorable side effects, such as cell proliferation inhibition and cell cycle inhibition on normal tissues as compared to irreversible inhibitors.

Claims

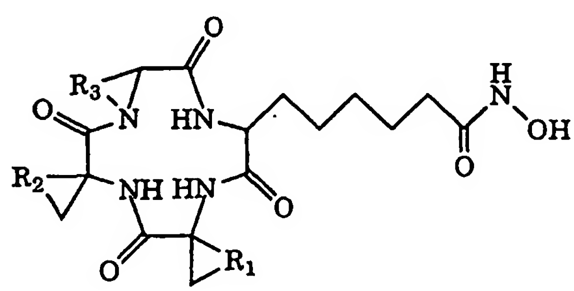
1. A cyclic tetrapeptide derivative represented by the following general formula (I), (I'), (I'') or (I''') or a pharmaceutically acceptable salt thereof:



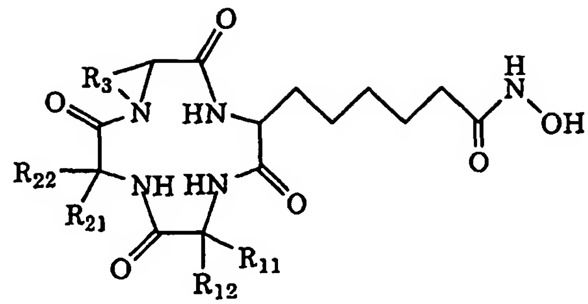
(I)



(I')



(I'')



(I")

wherein

each of R_{11}, R_{12}, R_{21} and R_{22} independently denotes hydrogen, a linear C_1-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached, or a branched C_3-C_6-alkyl group to which a non-aromatic cycloalkyl group or an optionally substituted aromatic ring may be attached; and

each of R_1, R_2 and R_3 independently denotes a linear C_1-C_5-alkylene group which may have a C_1-C_6 side chain, in which the side chain may form a condensed ring structure on the alkylene chain;

provided that at least one of R_{11}, R_{12}, R_{21} and R_{22} in general formula (I") is a cyclohexyl methyl group.

2. The cyclic tetrapeptide derivative according to claim 1, which is represented by said general formula (I), or a pharmaceutically acceptable salt thereof.

3. The cyclic tetrapeptide derivative according to claim 1, which is represented by said general formula (I'), or a pharmaceutically acceptable salt thereof.

4. The cyclic tetrapeptide derivative according to claim 1, which is represented by said general formula (I"), or a pharmaceutically acceptable salt thereof.

5. The cyclic tetrapeptide derivative according to claim 1, which is represented by said general formula (I"'), or a pharmaceutically acceptable salt thereof.

6. A histone deacetylase inhibitor comprising the cyclic tetrapeptide derivative or pharmaceutically acceptable salt thereof according to any one of claims 1 to 5 as an active ingredient.

7. An MHC class-I molecule expression-promoting agent comprising the cyclic tetrapeptide derivative or pharmaceutically acceptable salt thereof according to any one of claims 1 to 5 as an active ingredient.

8. A pharmaceutical composition comprising the cyclic tetrapeptide derivative or pharmaceutically acceptable salt thereof according to any one of claims 1 to 5 as an active ingredient.

9. The pharmaceutical composition according to claim 8, which is used as an anti-cancer agent.

Fig. 1

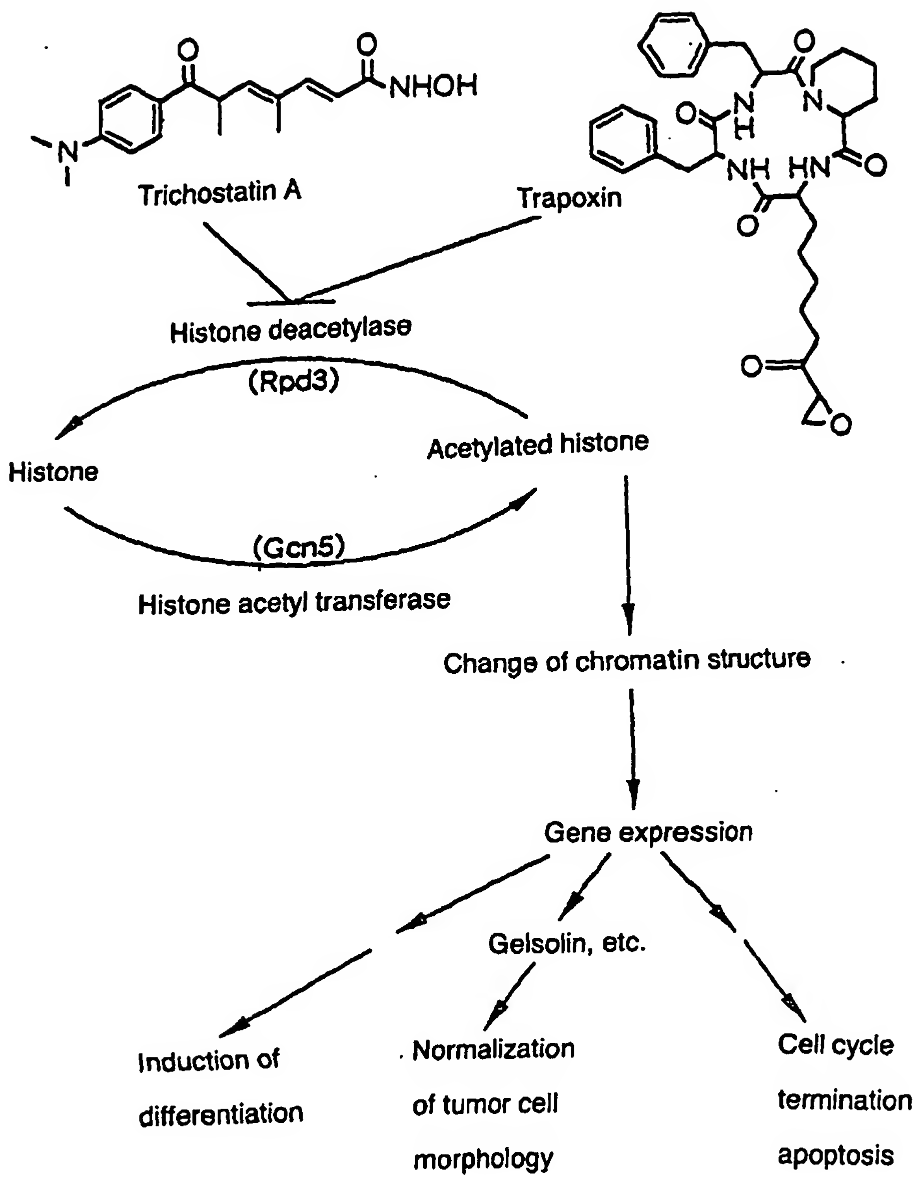
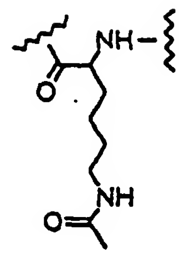
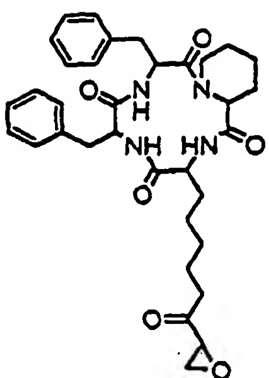

NHOH
Trichostatin A
Trapoxin
Histone deacetylase
(Rpd3)
Histone
Acetylated histone
(Gcn5)
Histone acetyl transferase
Change of chromatin structure
Gene expression
Gelsolin, etc.
Induction of differentiation
Normalization of tumor cell morphology
Cell cycle termination apoptosis

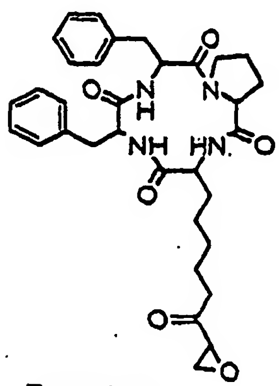
Fig. 2



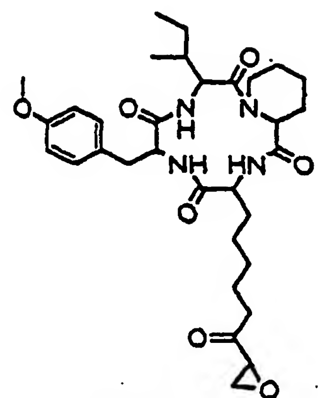
Acetylated lysine



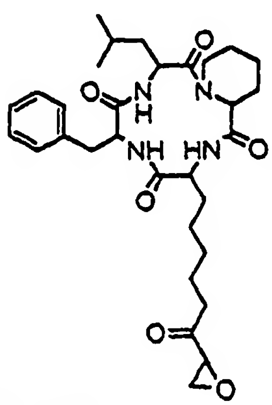
Trapoxin A



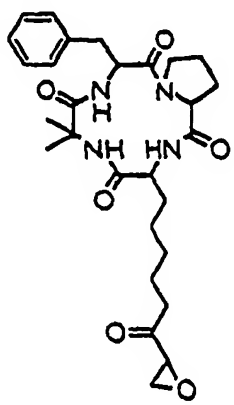
Trapoxin B



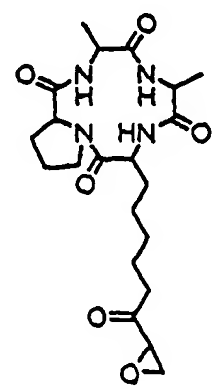
Cyl-2



WF-3161



Chlamydocin



HC-toxin

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/01141

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl^7 C07K 5/12, A61K 38/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl^7 C07K 5/12, A61K 38/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
REGISTRY(STN), CA(STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, 99/11659, A1 (JAPAN ENERGY CORPORATION), 11 March, 1999 (11.03.99) & AU, 9888885, A1	1-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 April, 2000 (21.04.00)	02.05.00

Name and mailing address of the ISA/	Authorized officer
Japanese Patent Office	
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)